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Clonality and Measurable Residual Disease (MRD) Testing on the Illumina® NextSeq™ Sequencers

Lymphoid and Myeloid Malignancies

INTRODUCTION

Next-generation sequencing (NGS) technology is rapidly transforming the paradigm of how gene rearrangement information can be used in the study of hematological malignancies. While traditional PCR-gel or PCR-capillary based gene rearrangement assays have limitations with samples containing low tumor burden or require expert data interpretation, NGS-based methods effectively address these limitations due to its superior sensitivity and specificity. The high resolution of NGS allows for more precise differentiation between clonal and polyclonal T-cell receptor (TCR) or immunoglobulin heavy chain (*IGH*) gene rearrangements and the identification of genomic mutations associated with *FLT3* and *NPM1* gene variants. Sequencing also provides accurate assessments of Somatic Hypermutation, which an important prognostic factor for CLL. NGS can uncover minute traces of malignant cells lingering after treatment, providing a significant advantage over traditional methods that might miss these low-burden populations. This translates to a heightened ability to detect measurable residual disease (MRD), with potential clinical applications including identification of early relapse and disease evolution as well as optimizing treatment strategies.

Benefits of using NGS for MRD detection include:

- Higher Sensitivity and Specificity: NGS allows for the detection of low-frequency mutations, enhancing diagnostic accuracy and the ability to monitor disease progression accurately.[1]

- Improved Clonality Assessment: With higher resolution and more objective interpretation, NGS overcomes limitations of gel or capillary based methods, such as the inability to differentiate clonal sequences from polyclonal background, leading to fewer false positives.[1]

- Quantitative Analysis: NGS enables precise assessments of clonality, Somatic Hypermutation and monitoring of disease burden over time, which is crucial for MRD assessment, developing treatment strategies, and predicting relapse.[1]

- Concordance with Established Methods: NGS-based assays show high concordance rates with traditional gel or capillary based methods demonstrating their clinical utility, comparable accuracy and higher specificity, particularly in diagnosing T-cell malignancies.[1,2]

- Enhanced Mutation Characterization: NGS facilitates detailed characterization of clonal populations, identification of intraclonal diversity and gene mutations, which may aid in prognostic assessments.

The National Comprehensive Cancer Network (NCCN) guidelines recommend MRD testing for several lymphoid and myeloid cancers, including multiple myeloma (MM), acute lymphoblastic leukemia (ALL), and chronic lymphocytic leukemia (CLL), and acute myeloid leukemia (AML).[3,4,5,6,7] European LeukemiaNet (ELN) guidelines recommend MRD testing following induction and consolidation courses to assess remission status and determine kinetics of disease response, and sequentially beyond consolidation to detect impending morphologic relapse.[8]

Over the past decade, Invivoscribe has pioneered the use of NGS to identify gene rearrangements and track residual disease over time. This innovation has led to the development of a suite of lymphoid and myeloid assays for use with Illumina's MiSeq™ and MiSeq™ NGS platforms, which provide target-specific sequences to aid hematologic disease research.

Today, the use of NGS technology for hematological disease research has become widely adopted, driving innovation for increased read depth, superior sequencing accuracy, streamlined data generation, and higher sample throughput without sacrificing cost.

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These technological improvements have created a high demand to adapt existing NGS assays for higher-throughput platforms, such as Illumina's NextSeq Systems. Here we present a performance comparison between the MiSeq and NextSeq 1000 platforms using identical libraries generated by our assays.

INVIVOSCRIBE NGS ASSAYS OVERVIEW

LymphoTrack® Assays for Lymphoproliferative Disorders

Invivoscribe's LymphoTrack® assays for MiSeq represent a significant improvement over PCR-based clonality assays due to their enhanced accuracy, specificity, and ability to provide detailed clonal information empower researchers with the information necessary to drive innovation and understanding of lymphoproliferative disorders. Therefore, these assays have three important and complementary uses:

1. Provides critical information on the existence of clonality.

2. Provides detailed sequence information on the degree of Somatic Hypermutation (*IGH* FR1 and IGHV Leader specific).

3. Identifies sequence information required to track clones in subsequent samples.

Primers included in the master mixes are designed with Illumina adapters, incorporating up to 24 different indices. This design provides a streamlined approach consisting of a single-step PCR and the ability to pool amplicons from several different samples and targets into one sequencing run for additional cost efficiencies. Assay interpretation and analysis is completed by the associated RUO LymphoTrack Software, which generates objective reports and data visualization.

Extending application utility, LymphoTrack Assays can also be used to identify and track measurable residual disease (MRD) in lymphoproliferative diseases.

LymphoTrack MRD Solutions can be used to track levels of MRD in subjects following therapy and throughout remission.

As part of the LympoTrack MRD Solution, LymphoTrack Low Positive Controls are used as an external quality control for each run, while LymphoQuant® Internal Controls are spiked into each sample to calculate clonal cell equivalents. These RUO DNA controls are designed for use with LymphoTrack Assays and LymphoTrack MRD Software to track clonal sequences.

The LymphoTrack MRD Bundled Solutions are offered for T- or B-Cell assessment enabling researchers to accurately detect and trend clonal evolution in longitudinal studies with unprecedented simplicity. In summary, LymphoTrack MRD Solutions offer improved sensitivity, objectivity, specificity, and quantitative capabilities compared to traditional methods, making them a promising tool for future clinical applications in lymphoproliferative diseases.

MRD NGS Assays for Myeloid Diseases

In addition to the LymphoTrack assays, Invivoscribe's NGS products now includes two pivotal Research Use Only (RUO) measurable residual disease assays for AML. Our innovative *FLT3* ITD MRD Assay (RUO), targets the FMS-like tyrosine kinase 3 (*FLT3*) gene which encodes a receptor tyrosine kinase that is normally expressed on many cell types, including hematologic stem cells. Mutation of the *FLT3* receptor, by internal tandem duplication (ITD) of the juxtamembrane domain, causes constitutive activation of the *FLT3* receptor. *FLT3* ITD mutations are present in about 25% of patients with AML and are characterized by an increased risk of relapse.

The *NPM1* MRD Assay (RUO), targets the Nucleophosmin (*NPM1*) gene which encodes for a protein involved in cellular activities that may relate to proliferative and growth-suppressive roles in the cell. As one of the most commonly mutated genes in AML, *NPM1* gene mutations occur in about one-third of the cases of primary AML. Of the subjects with an *NPM1* mutation at diagnosis, roughly 50% relapse during the first 3 years, particularly those with a concurrent *FLT3*-ITD mutation.[9]

Recent research suggests the presence of ITD and *NPM1* mutations can be indicative of disease outcome. In a study evaluating the persistence of residual *FLT3*-ITD and *NPM1* mutations among subjects with AML in first remission prior to allogeneic cell transplant, those with an allele fraction ≥0.01% trended towards elevated risk of relapse and mortality compared to those without these mutations.[10]

Each MRD assay kit includes 24 uniquely indexed master mixes, allowing multiple samples and targets to be sequenced on the same flow cell improving cost efficiency. Assay interpretation and data analysis are completed with either the *FLT3* ITD MRD Software or *NPM1* MRD Software in under one hour, providing sequence annotations and objective results.

NGS INSTRUMENT OVERVIEW

Illumina MiSeq™

The Illumina MiSeq is a benchtop sequencer designed for targeted sequencing applications. It is known for its high accuracy and relatively low throughput, making it suitable for small to medium-sized laboratories.

Metric Description

Sequencing Reads and Length: Depth Capable of generating up to 50 million paired-end reads per run, with a read length of up to 2x300 bp.

Quality: >70% bases higher than Q30 for 2x300 bp.

TAT: Approximately 56 hours per run, depending on the assay, kit, and workflow being performed.

Cost-Effectiveness: Cost per run is relatively low, making it economical for laboratories with moderate sample volumes.

Illumina NextSeq 1000™

The NextSeq 1000 is a recent addition to Illumina's NGS portfolio, offering higher throughput and more flexibility compared to the MiSeq. It is designed to support a broader range of applications, from targeted sequencing to whole-exome sequencing.

Metric Description

Sequencing Reads and Length: Depth Capable of generating up to 3.6 billion paired-end reads per run, with a read length of up to 2x300 bp.

Quality: ≥85% bases higher than Q30 for 2x300 bp.

TAT: Faster than MiSeq, with runs completing in approximately 34 hours depending on the assay, kit, and workflow being performed.

Cost-Effectiveness: Higher initial cost per run, but more cost-effective for high throughput needs due to the larger number of samples that can be processed simultaneously.

MATERIALS AND METHODS

Positive DNA from cell lines or clinical samples were contrived using negative DNA as a background to create panels for each assay. The evaluated assays included V-J rearrangement (NGS clonality) assays for immunoglobulin heavy chain *IGH* (IGHV Leader, FR1, FR2 and FR3), immunoglobulin light chain (*IGK*), T-cell receptor gamma (*TRG*), T-cell receptor beta (*TRB*), and MRD assays for *FLT3* internal tandem duplications and *NPM1* mutations. The workflow for Invivoscribe's NGS assays is shown below in Figure 1.

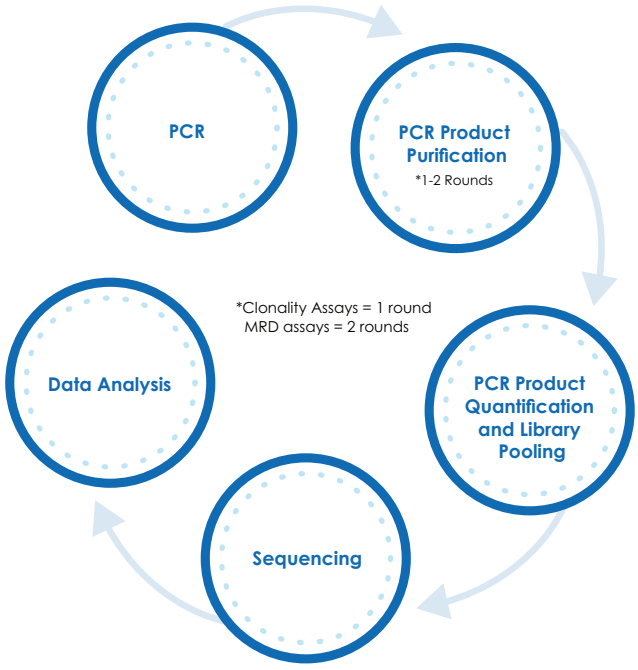

Figure 1: Workflow for Invivoscribe's NGS Assays

Three pooled libraries were generated:

Library A: comprised of seven V-J Clonality Rearrangement Assay Samples
Library B: comprised of *FLT3* MRD Assay Samples
Library C: comprised of *NPM1* MRD Assay Samples

Library A was sequenced at 2x301 cycles on the MiSeq with a loading concentration of 18 pM using the MiSeq Reagent Kit v3 and on the NextSeq 1000 with a loading concentration of 650 pM and 12.5% PhiX using NextSeq 1000/2000 P1 reagents, respectively. FASTQ files from both platforms were analyzed using the same in-house developed LymphoTrack Dx Software - MiSeq (v2.4.3). Top percent reads from each of the target specific V-J rearrangements were compared between the NGS platforms.

Libraries B (*FLT3* ITD) and C (*NPM1*) were sequenced separately each at 2x301 cycles on the MiSeq with a loading concentration of 14 pM and 12.5% PhiX using the MiSeq Reagent Kit v3 and sequenced together in a combined library on the NextSeq 1000 with a loading concentration of 650 pM and 12.5% PhiX using NextSeq 1000/2000 P2 reagents.

FASTQ files from both platforms were analyzed using the same in-house developed *FLT3*-ITD MRD Software (v1.2) and *NPM1* MRD Software (v1.1.1). Detected variant read frequencies (VRF) from each of the target specific mutations were compared between the NGS platforms.

Table 1: Illumina Materials

Vendor	Description	Catalog #
[A]Illumina	MiSeq Reagent Kit v3	MS-102-3003
[B]Illumina	NextSeq 1000/2000 P1 Reagents	20075294
[C]Illumina	NextSeq 1000/2000 P2 Reagents	20075295

RESULTS

Clonality Assays

The full suite of Invivoscribe's clonality NGS assays includes 4 targets within the *IGH* gene (Leader, FR1, FR2, and FR3), as well as assays for the *IGK*, *TRG*, and *TRB* genes. Each of these were combined into a single amplification library which was run on both the MiSeq and the NextSeq 1000. This library generated 32 million pass filter (PF) reads with the MiSeq and generated 161 million PF reads with the NextSeq 1000, 5-fold more PF reads as compared to MiSeq. A comparison of the top percent reads generated from both instruments yielded relative R^2 of the correlations all greater than 0.99. Each assay's relative R^2 values are listed in Table 2.

Table 2: Clonality Assay Results Correlation between Instruments

Assay	Correlation (R^2)	Key Run Metrics	
		MiSeq	NextSeq 1000
IGH Leader	0.997	PF: 32 million Q30: 77.02% Sequencing time: ~56 hours	PF: 161 million Q30: 86.86% Sequencing time: ~34 hours
IGH FR1	0.998		
IGH FR2	0.999		
IGH FR3	0.999		
IGK	0.999		
TRG	0.999		
TRB	0.996		

Plotting the results from each clonality assay to compare the instruments is depicted in Figures 2 & 3.

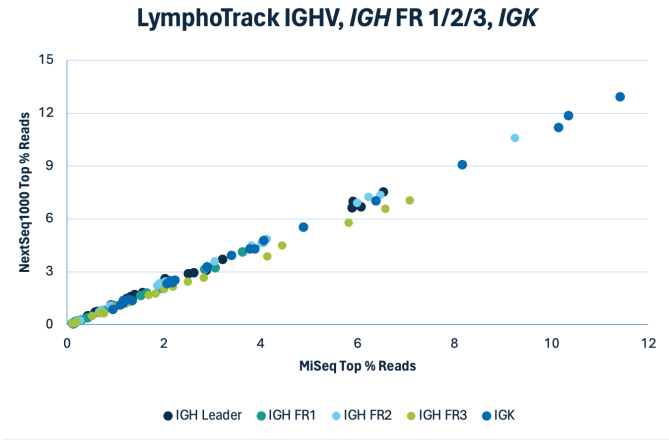

Figure 2: IGHV, *IGH* FR1/2/3 & *IGK* Clonality Comparison

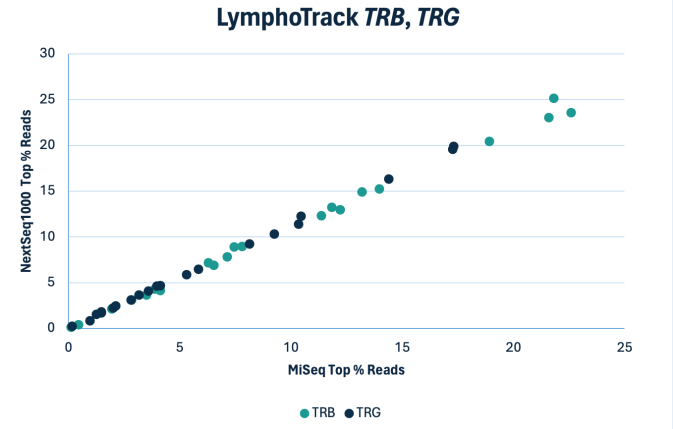

Figure 3: *TRB, TRG* Clonality Comparison

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MRD Assays

Invivoscribe's myeloid MRD assay menu includes the *FLT3* ITD MRD Assay and *NPM1* MRD Assay. Separate libraries were generated for each target (*FLT3* ITD and *NPM1*) and sequenced individually on the MiSeq to ensure a suitable read depth was achieved. The MiSeq generated 19 million PF reads with the *FLT3* ITD MRD library, and 14 million PF reads with the *NPM1* MRD library.

The same libraries were then combined and sequenced on the NextSeq 1000, resulting in 11-fold more PF reads (413 million), as compared to the two MiSeq runs. The results generated from both instruments were compared and the relative R^2 of the variant read frequencies (VRF) correlation for each were greater than 0.999. Each assay relative R^2 values are listed in Table 3.

Table 3: MRD Assay Results Correlation between Instruments

Assay	Correlation (R^2)	Key Run Metrics	
		MiSeq	NextSeq 1000
FLT3 ITD MRD	0.999	PF: 19 million Q30: 89.00% Sequencing time: ~55 hours	PF: 413 million Q30: 89.63% Sequencing time: ~44 hours
NPM1 MRD	1.000	PF: 14 million Q30: 89.94% Sequencing time: ~56 hours	

Plotting the results from each MRD assay to compare the instruments is depicted in Figure 4.

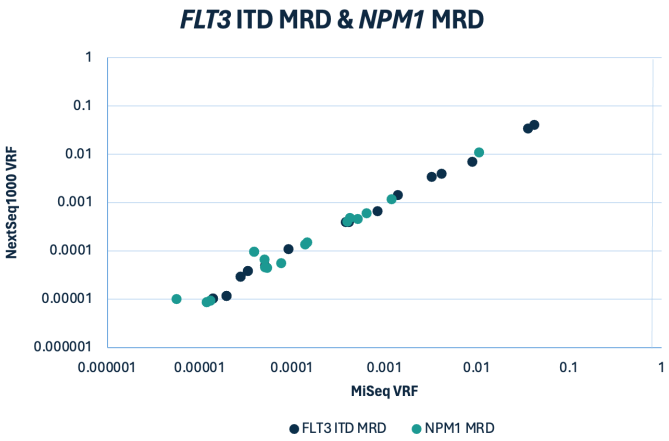

Figure 4: *FLT3* ITD MRD & *NPM1* MRD Comparison

The NextSeq 1000 can achieve even greater sensitivity for MRD detection due to its deeper sequencing capability, potentially obtaining sensivitity levels as low as 10^{-6}.

This allows more precise detection of low-frequency clones, which can be observed when comparing detection levels to those achieved by the MiSeq, as exhibited in Table 4 for *FLT3* ITD MRD and Table 5 for *NPM1* MRD.

Table 4: *FLT3* ITD MRD Detection Results

ITD Insertion Length	VRF Target	MiSeq (VRF)	NextSeq 1000 (VRF)
Medium Insert: 42 bp	5.00E-02	3.56E-02	3.51E-02
	5.00E-03	3.23E-03	3.46E-03
	5.00E-04	4.11E-04	3.96E-04
	5.00E-05	3.28E-05	3.88E-05
	1.00E-06	NOT DETECTED	NOT DETECTED
Long Insert: 172 bp	5.00E-02	1.39E-03	1.45E-03
	5.00E-03	9.04E-05	1.11E-04
	5.00E-04	1.93E-05	1.18E-05
	5.00E-05	**NOT DETECTED**	**1.34E-06**
	1.00E-06	NOT DETECTED	NOT DETECTED

Table 5: *NPM1* MRD Detection Results

NPM1 Mutation Type	VRF Target	MiSeq (VRF)	NextSeq 1000 (VRF)
Type: A	1.00E-05	1.30E-05	9.55E-06
	1.00E-06	NOT DETECTED	NOT DETECTED
Type: B	5.00E-05	1.18E-05	8.74E-06
	1.00E-05	**NOT DETECTED**	**2.27E-06**
Type: D	5.00E-05	5.03E-05	4.59E-05
	1.00E-05	5.57E-06	1.03E-05
Type: Other	5.00E-05	3.86E-05	9.68E-05
	1.00E-05	**NOT DETECTED**	**1.62E-06**

CONCLUSION

Invivoscribe's NGS assays demonstrate high concordance between the MiSeq and NextSeq 1000 platforms for both clonality and MRD detection. Both platforms yielded highly correlated results ($R^2 > 0.99$) for all clonality and MRD assays, indicating reliable detection of gene rearrangements and mutations.

These results demonstrate that Invivoscribe's RUO LymphoTrack MiSeq and AML NGS MRD assays are compatible with the NextSeq 1000.

Moreover, the increased read depth of the NextSeq 1000 platform enables pooling more libraries for sequencing at a greater cost efficiency as well as enhanced sensitivity, exhibited by the detection of 3 dilution levels missed by the MiSeq. This provides evidence of enhanced sensitivity, which is important when assessing residual disease.

However, the NextSeq 1000 offers a significant advantage in throughput. During this investigation, the NextSeq 1000 generated a substantially greater number of reads compared to the MiSeq (up to 5-fold for clonality and 11-fold for MRD). This translates to:

- **Potential for increased sensitivity:** Improved detection of low-frequency clones - particularly crucial for MRD analysis where identifying even minute traces of residual disease is critical;
- **Enhanced confidence in negative results:** a deeper sequencing run translates to a higher number of reads analyzed. This can lead to greater confidence in negative MRD results, potentially reducing the risk of false negatives and unnecessary treatment interventions; AND
- **Improved efficiency:** While the difference is modest, the NextSeq 1000 demonstrated slightly faster run times for both clonality and MRD assays. This can contribute to improved workflow efficiency, allowing laboratories to process more samples and potentially reduce turnaround times.

Invivoscribe's NGS assays offer reliable performance on both platforms. However, for laboratories seeking to optimize workflow efficiency, unlock the potential for increased sensitivity in MRD detection, and gain deeper sequencing insights, the NextSeq 1000 emerges as the preferred platform. Its enhanced sequencing depth can empower clinicians with more precise information to guide personalized treatment decisions for their patients.

ABOUT INVIVOSCRIBE

Invivoscribe is a global, vertically integrated biotechnology company dedicated to Improving Lives with Precision Diagnostics®. For nearly thirty years, Invivoscribe has improved the quality of healthcare worldwide by providing high quality standardized research reagents, tests, and bioinformatics tools to advance the field of precision medicine. Invivoscribe has a successful track record of partnerships with global pharmaceutical companies interested in developing and commercializing companion diagnostics, and provides expertise in both regulatory and laboratory services. Providing distributable reagents and kits, as well as clinical trial services through its globally located clinical lab subsidiaries (LabPMM), Invivoscribe is an ideal partner from proof of concept through commercialization.

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Invivoscribe's RUO NGS Assays and Software for use with Illumina Sequencers

Catalog #	Product Description	Quantity
71210009	LymphoTrack *IGH* FR1 Assay Kit A – MiSeq	8 indices – 5 reactions each
71210039	LymphoTrack *IGH* FR1 Assay Panel – MiSeq	24 indices – 5 reactions each
71210149	LymphoTrack *IGH* FR1 Assay Panel B – MiSeq	24 indices – 5 reactions each
71210089	LymphoTrack *IGH* FR2 Assay Kit A – MiSeq	8 indices – 5 reactions each
71210099	LymphoTrack *IGH* FR2 Assay Panel – MiSeq	24 indices – 5 reactions each
71210109	LymphoTrack *IGH* FR3 Assay Kit A – MiSeq	8 indices – 5 reactions each
71210119	LymphoTrack *IGH* FR3 Assay Panel – MiSeq	24 indices – 5 reactions each
71210129	LymphoTrack *IGH* FR1/2/3 Assay Kit A – MiSeq	8 indices per FR region– 5 reactions each
71210139	LymphoTrack *IGH* FR1/2/3 Assay Panel – MiSeq	24 indices per FR region – 5 reactions each
71210059	LymphoTrack IGHV Leader Somatic Hypermutation Assay Kit A – MiSeq	8 indices – 5 reactions each
71210069	LymphoTrack IGHV Leader Somatic Hypermutation Assay Panel – MiSeq	24 indices – 5 reactions each
71220009	LymphoTrack *IGK* Assay Kit A – MiSeq	8 indices – 5 sequencing reactions each
71220019	LymphoTrack *IGK* Assay Panel – MiSeq	24 indices – 5 sequencing reactions each
72250009	LymphoTrack *TRB* Assay Kit A – MiSeq	8 indices – 5 reactions each
72250019	LymphoTrack *TRB* Assay Panel – MiSeq	24 indices – 5 reactions each
72270019	LymphoTrack *TRG* Assay Kit A – MiSeq	8 indices – 5 reactions each
72270009	LymphoTrack *TRG* Assay Panel – MiSeq	24 indices – 5 reactions each
14120019	*FLT3* ITD MRD Assay	96 Reactions
14160019	*NPM1* MRD Assay	96 Reactions
S100003	LymphoTrack Enterprise Software	1 software package
S100005	*FLT3* ITD MRD Software	1 software package
S100004	*NPM1* MRD Software	1 software package

To learn more about Invivoscribe and LabPMM, **visit us online at invivoscribe.com** or **call us at +1.858.224.6600**.

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